Introduction

The genus Citrus of the family Rutaceae, also known as citrus fruit family, comprises of 100 species, many of which are probably hybrid (Ghazanfar *et al.,* 1980). The loose-skinned mandarin orange (*Citrus reticulata* Blanco) is one of the most economically important and popular fruits in the world, constituting about 41% of the total citrus fruits produced in India. The northeastern Himalayan region is one of the three major centres of mandarin orange cultivation in India. About 1600 ha, distributed over nine states -Assam, Arunachal Pradesh, Meghalaya, Manipur, Mizoram, Nagaland, Tripura, Sikkim and the Darjeeling district of West Bengal) is under mandarin orange cultivation, where this high-value crop contributes substantially to the region's small farm economy (Ghosh and Singh 1993). The nutritional value of citrus fruits is well known in our dietary requirements. Presently, it is the third largest fruit industry after mango and banana in India. The North-Eastern (NE) region of India offers favourable climatic conditions for cultivation of various citrus species. Submountain and hilly tracts of states like Meghalaya, Assam, Manipur, Arunachal Pradesh, Mizoram, Nagaland, Tripura, Sikkim and Darjeeling District, West Bengal grow excellent quality citrus fruits. Different citrus species, viz. mandarin, sweet orange, lemon and other limes are cultivated in all the states of the NE region covering 57.2 thousand hectares, with a total production of 306 thousand tones (Mallick *et al.*, 2006).

Citrus reticulata (Blanco) is the principal fruit crop of Darjeeling hills known as 'Darjeeling mandarin' (Plate 1). It is considered as the most important cash crop of the marginal families of the hills (Thapa, 2007). But recently it's decline is posing a threat to the sustainability of citriculture and the stakeholders respectively (Chakraborty *et al.*, 2011a,b). Citriculture in the hilly terrain is undertaken as secondary and tertiary source of income. However, extensive cultivation is seldom undertaken owing to the topography of the terrain. This has resulted in establishment of accountable smaller orange grooves or orchards. Moreover, in the recent past the area under cultivation as well as the production has declined. This can be attributed to various parameters ranging from on-field cultural practices, quality of planting materials, occurrence of diseases that may be due to viruses, fungi, insects or bacteria, and soil quality. The entire citrus orchards in NE India are of seedling origin, with few budded or grafted plants at some experimental research stations. Like many other crops, citrus in this region is plagued with a host of diseases caused by different

Plate 1 (figs. A-B): ***Citrus reticulata*** **in Mirik orchard [A] and harvested mandarin fruits in market place for sale [B].**

The distribution or localization of diseased orchards throughout the hilly slopes of Darjeeling hills is random or mosaic in pattern. The severity of the condition prevalent in the orchards cannot be attributed to one particular disease or factor. The mandarin cultivation in Darjeeling has a massive decline due to various pathological, entomological and nutritional stresses. (Mukhopadhyay *et al*, 1996)

The evident symptoms that can be observed are defoliation of young shoots and twigs and ultimate drying up of the whole plant. The symptomlogy, hence observed is indicative of the orange orchards being affected by "citrus dieback" disease. A multidisciplinary attempt has been made to assess the conditions of the orchards and analyse the relative abundance of the contributing factors for the citrus decline (Mukhopadhyay *et al.*,1985). Lack of proper knowledge on agro-techniques of orchard management of citrus cultivation in terms of nursery management, proper seedling selection, soil health status, plant protection measures, amount of manuring and fertilizer required for the plant, irrigation in winter, mulching practice, pruning practice, intercropping (suitable and unsuitable crop) etc. after the establishment of orchard singly or in combination has lead to citrus decline in Darjeeling hills. It was observed that chronic infection of foot and root rot alone or coupled with the infection of *Fusarium* and *Macrophomina* were associated to large number of dieback plants in combination with nutrient deficiencies. It is a well-known fact that heavy phosphorus application causes zinc deficiency in plant tissue (Marschner, 1995; Cooper and Tinker, 1978). Lambert *et al.* (1979) showed that high phosphorus depressed mycorrhizal inoculation, thus plants were not able to get more zinc.

Biological control is generating a great enthusiasm to play a role in sustainable agriculture. Biological control agents act as biofertilizers or as bio pesticides. Biofertilizers are the preparation containing cells of microorganism which may be nitrogen fixers, phosphorus solubilizers or organic matter decomposers. In short, they are called as bio inoculants which on supply to plants improve their growth and yield. Mycorrhizal fungi provide an effective alternative method of disease control especially those pathogens which affect the below ground plant parts. In mycorrhizal fungi lies enormous potential for use as biocontrol agent for soil borne diseases as the root diseases are one of the most difficult to manage and lead to loss in disturbing proportions. There is evidence that root colonization by Glomalean fungi can reduce disease severity in hosts affected by *Phytopthora parasitica* and other root pathogens (Davies and Mange, 1981). Since more than one Glomalean fungus can occupy a system and according Reed *et al.* (1985) root systems may benefit from the differential resource acquisition of several Glomalean fungi simultaneously. Dual or triple action with phosphate solubilizing microorganisms (PSM) such as species of *Pseudomonas, Bacillus, Flavibacterium* and *Arthrobacter* have been found to increase the effectiveness of the VAM fungi. PSM produce, mono, -di-, and tri-carboxylic acids which helps in dissolving mineral phosphate by Fe and Al ions. The effect of environmental factors like pH (Reed *et al.*, 1976; Abbot and Robson,1985), light, temperature, moisture, associated microorganisms (Graham *et al.*, 1981) and plant genotype has been well studied. Evidence exist that mycorrhizal plants are capable of resisting the parasitic invasion and minimize the loss caused by soil borne pathogens. Bio-control of *Fusarium* wilt of cotton, jute, tomato and *Macrophomina* root rot of cowpea was achieved by the application of VAM fungi. Ability of an isolate of *Trichoderma harzianum* and arbuscular mycorrhizal fungi (AMF) in enhancing growth of tomato seedlings and control of a wilt pathogen caused by *Fusarium oxysporum* f.sp. *lycopersici* was demonstrated by Mwangi *et.al* (2011).

The dynamism of rhizosphere dwelling bacteria can have a profound effect on plant health and its surrounding environment. Rhizosphere colonization is important not only as the first step in pathogenesis of soil borne microorganisms , but also is crucial in the application of microorganisms for beneficial purposes; most significant among these applications are biofertilization, phytostimulations, biocontrol and phytoremediation (Lugtenberg *et al.*, 2001). The prospect of manipulating crop rhizosphere microbial populations by inoculation of beneficial bacteria to increase plant growth has shown considerable promise in laboratory and greenhouse studies, but responses have been variable in the field. Although non chemical control of pests and diseases seems an attractive option in sustainable agricultural systems, its practical application is severely constrained by lack of reproducible results in comparison to the use of agrochemicals. The biotic and abiotic factors in the environment, which influence the efficiency of pathogen suppression, are not well understood. Nevertheless it is known that some soil become suppressive to fungal, bacterial and nematode pests when susceptible host plants are grown continuously for several years and there is evidence that this is due to the development of soil biological communities which inhibit pests survival or infection of the hosts

Little is known about the microbial endophytic community of citrus (Araujo *et al.*, 2001) and the possible impact of endophytes on yield and especially on the control of citrus plant diseases, making the isolation and study of these microorganism for morphological physiological and genetic studies. A number of fungi are known to colonize plant roots but do not cause disease. These include Mycorrhizas, binucleate *Rhizoctonia* spp., *Piriformaspora indica,* various plant growth promoting rhizobacteria and *Trichoderma* spp. (Shoresh *et.al*, 2010). Many of these organisms have been known for decades as agents as agents that biocontrol plant diseases, but recent studies have demonstrated that they have many other attributes. These organisms are very clearly endophytic multifunctional plant symbionts (Harman, 2011a). Typically, the fungi penetrate the outer layers of the epidermis and plant cortex, and establish chemical communication with the plant. An initial result is that the fungi are walled off by the plant, but not killed. A relatively large number of the chemical communicants (effectors/elicitors) released by the fungi are known; these include small proteins, peptides and other metabolites, including volatile ones. It is also remarkable that qualitatively similar effects are induced in plants by a variety of plant-associated root colonizing microbes, including plant growth-promoting rhizobacteria (PGPR) and mycorrhizal fungi. Presumably, the ability of these microbes to induce changes in plants, resulting in a large number of healthy roots in which they live, provides a competitive advantage (Harman, 2011b).

The present investigation was undertaken taking into consideration many factors but the focus was mainly on fungal pathogens. The reason being that the on field observation as related to the symptomology on preliminary assessment indicated that the degree severity was more due to fungal pathogens.The goal of this investigation as perceived was to initiate an understanding of the dynamics of micro-flora dwelling in the rhizosphere of the citrus plants and their state of interaction with prevailing pathogens affecting the citrus plants. The present study was designed with an aims to explore the possibility of using beneficial bioinoculant, plant growth promoting rhzobacteria (PGPR) and arbuscular mycorrhizal fungi (AMF) isolated from rhizosphere of mandarin plants (*Citrus reticulata*), specially Darjeeling variety grown in Darjeeling and Sikkim hills and to develop a formulation with bioinoculants for management of root rot disease caused by fungal pathogen (*Macrophomina phaseolina*).

To evaluate and corroborate the goal certain objectives were outlined:

- Investigation on prevalent root diseases of mandarin orange orchards of Darjeeling Hills.
- Screening of Arbuscular mycorrhizal (AM) fungi and other beneficial micro-organisms from rhizosphere of mandarin plants.
- *In vitro* interaction study among frequently associated rhizosphere microorganisms and root pathogens.
- Serological and molecular detection of dominant root pathogen.
- RAPD-PCR and phylogenetic analysis of microorganisms of mandarin rhizosphere.
- Selection and molecular identification of bioinoculants
- Evaluation of bioinoculants for suppression of root rot disease of mandarin
- Activation of defense response of mandarin plants following application of bioinoculants against root rot pathogen and assocaited changes in defense enzymes.
- Cellular location of defense enzyme in root and leaf tissues of mandarin plants following induction of resistance.